Neurofilament-Like Immunoreactivity in the Sea Anemone *Condylactis gigantea* (Cnidaria: Anthozoa)

CHRISTIAN DELLACORTE[1]*, DON S. ANDERSON[2],
WILLIAM. O. McCLURE[1], AND D. LYNN KALINOSKI[3]

[1]*Department of Neurobiology, University of Southern California, Los Angeles, California
90089-2520;* [2]*School of Dentistry, University of Southern California, Los Angeles, California 90089;*
[3]*Monell Chemical Senses Center, 3500 Market St., Philadelphia, Pennsylvania 19104*

Abstract. The neuronal cytoskeleton contains neurofilament proteins that serve as markers for nervous tissue in many species across phyla. Antiserum generated to mammalian neurofilaments was used for immunocytochemical staining of tissues in the sea anemone *Condylactis gigantea* (Cnidaria: Anthozoa). Specific staining, visible at the light and electron microscope levels, was found in the tissues of the tentacle. Proteins were extracted from the tissues and solubilized. SDS-polyacrylamide gel electrophoresis and Western blotting revealed two bands of MW_r 156 kD and 74 kD that reacted with antiserum generated to neurofilaments. The protein bands also bound a monoclonal antibody shown to react with a highly conserved epitope in many classes of intermediate filaments. These data suggest that the neurons of this anthozoan contain neurofilament-like proteins with molecular properties similar to those of mammalian neurons.

Introduction

Nervous systems probably evolved in animals related to members of present-day cnidarians. The nervous system in these primitive metazoans has been described as consisting of diffuse nets of sensory, motor, and neurosecretory cells that provide connections within the epithelial layers (Bullock and Horridge, 1965).

Neuronal cells and processes contain a filamentous framework that forms the cytoskeleton. Neurofilaments (NFs), a class of structures that express a head-rod-tail organization, belong to the family of intermediate fila-

ments (IFs) and are major components of the neuronal cytoskeleton (Wang *et al.*, 1985; Steinert and Roop, 1988). The central helical rod domain has a hydrophobic backbone that causes aggregation of the subunits into coiled-coil 10-nm filamentous structures; this domain also contains an evolutionarily conserved region, a major feature found in identified IF proteins (Pruss *et al.*, 1981; Geisler and Weber, 1982; Hanukoglu and Fuchs, 1983; Steinert *et al.*, 1983). Besides playing a central role in the structural matrix of neurons, NFs are of particular interest because they may be determining factors in the development of the central nervous system.

The presence and location of these filaments have been visualized by various methods, including histological staining and immunocytochemistry (Phillips *et al.*, 1983; Lasek *et al.*, 1985). NFs have been isolated from many cell types (Cooke and Chase, 1971; Daniels and Huneeus, 1973; Brown *et al.*, 1976) and a variety of species (Lasek *et al.*, 1979; Moon *et al.*, 1981; Phillips *et al.*, 1983). Mammalian neurofilaments are composed of three polypeptides (the "triplet" proteins), with molecular weights of approximately 200 kD (NF-H), 160 kD (NF-M), and 70 kD (NF-L) (Kaufmann *et al.*, 1984; Scott *et al.*, 1985). Some non-mammalian species have triplet proteins that are homologous to those found in mammals. Others, however, have only a single high molecular weight subunit that is immunologically similar to both NF-H and NF-M (Shaw *et al.*, 1984; Shaw, 1991). In invertebrates, neurofilaments are even more variable than their vertebrate counterparts (Eagles *et al.*, 1981; Shaw, 1991; Szaro *et al.*, 1991; Way *et al.*, 1992).

The cytoskeletal components that form an early metazoan nervous system, such as the one found in members of the phylum Cnidaria, are not well-defined. We use im-

munocytochemical techniques, as well as protein isolation, light microscopy, and transmission electron microscopy (TEM), in our studies of the nervous system in these lower organisms. In this investigation, we focused on confirming the presence of neurofilament-like antigens in the tissues of the subtropical sea anemone *Condylactis gigantea* (Cnidaria: Anthozoa). Our studies suggest that neurons of this anthozoan contain neurofilament-like proteins with molecular properties similar to those of higher species.

Materials and Methods

Animals

Specimens of *Condylactis gigantea* were obtained from a supplier in Los Angeles, California, and kept in aerated, filtered, recirculating aquaria at 24°C in natural seawater from the Catalina Marine Science Center (Santa Catalina Island, California). Animals acquired from local suppliers in Philadelphia, Pennsylvania, were kept in aerated, filtered artificial seawater. All animals were maintained on a 12/12 photoperiod and fed *Artemia* nauplii, small pieces of squid, or both three times a week. Water was changed at two-week intervals.

Specimens were relaxed by immersion in seawater containing succinylcholine chloride (67 μg/ml, Sigma Chemical Co., St. Louis, Missouri). The tentacles of these relaxed animals were of about normal length, but the diameter was somewhat smaller than normal. They were clamped with a hemostat at the junction of the oral disk and excised.

Bodian silver stain

The Bodian silver stain method allows visualization of neural tissue in many species (Bodian, 1936; Gambetti *et al.*, 1981). Bodian silver stain was applied to tissue sections as described by Clark (1981) and to SDS polyacrylamide gels after electrophoresis of proteins as described by Gambetti *et al.* (1981).

Light microscopic immunocytochemistry

Excised tentacles were placed in 2% paraformaldehyde in phosphate buffered saline (PBS; 0.1 M sodium phosphate buffer, 0.9% NaCl), pH 6.5, for 2 h at room temperature and were postfixed in 2% paraformaldehyde in PBS, pH 11 (Berod *et al.*, 1981), for 8 h at 4°C. Samples were rinsed three times in PBS, pH 7.4, and cryoprotected by immersion for 12 h each in 10, 20, and 30% sucrose in PBS, pH 7.4, at 4°C. Tissue was placed in Tissue-Tek (Miles Inc., Elkhart, Indiana) and frozen in isopentane at liquid nitrogen temperatures. Twenty micrometer sections were made on a Microm 500 M Cryostat (Zeiss Inc.,

Thornwood, New York) at −20°C and thaw-mounted on slides.

Slides were placed in PBS for 20 min and endogenous peroxidases were inactivated with 0.3% hydrogen peroxide in PBS for 20 min. After being rinsed in three successive 15-min changes of PBS, the specimens were incubated for 1 h in PBS containing 0.3% Triton X-100 and 10% normal serum (Vector Laboratories, Burlingame, California), then placed in primary antiserum [polyclonal neurofilament 200 (NF 200, Chemicon International, Temecula, California, and Sigma Chemical Co., St. Louis, Missouri)] at a 1:20,000 dilution for 12 h at 4°C. Three 15-min rinses in PBS were followed by incubation with a biotinylated secondary antibody, several PBS rinses, and incubation with a preformed avidin/biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, California) following the manufacturer's instructions. Tissues were rinsed two successive times for 10-min each in freshly made acetate/imidazole buffer (0.01 M imidazole, 0.05 M sodium acetate, pH 7.4) and transferred to a nickel chromagen [0.095 M nickel (II) sulfate, 0.05% DAB, 0.03% hydrogen peroxide] in acetate/imidazole buffer to develop.

Control procedures included deletion of secondary antibodies and replacement of the primary antiserum with normal serum.

Electron microscopic immunocytochemistry

Portions of tentacles were placed in 4.0% paraformaldehyde/0.5% glutaraldehyde in 0.1 M PBS, pH 6.5, for 1 h at room temperature and transferred to 4.0% paraformaldehyde/0.5% glutaraldehyde, pH 11, for 8 h at 4°C before washing in PBS, pH 7.4, and dehydration in a graded ethanol series. Samples were infiltrated and embedded in EM-BED-812 (Electron Microscopy Sciences, Ft. Washington, Pennsylvania) according to manufacturer's directions. Silver to gold sections were placed on nickel grids (Ted Pella Inc., Redding, California). Grids were floated on small amounts of reagent at room temperature as follows: (1) 1 h on 0.1 M PBS at pH 7.4; (2) 5 min on a saturated solution of sodium metaperiodate, followed by 1 h on several changes of PBS; (3) 1 h on 10% normal serum (Vector Laboratories, Burlingame, California); (4) three successive 30-min rinses on PBS; and (5) 2 h on antiserum to polyclonal NF 200 (Sigma Chemical Co., St. Louis, Missouri). Grids were rinsed on three successive changes of PBS and floated on a 1:40 dilution of gold-conjugated IgG (Janssen Life Sciences Products, Piscataway, New Jersey) for 1 h, followed by two successive 20-min distilled water rinses. Grids were postfixed for 15 min on a drop of 2% glutaraldehyde and poststained in lead citrate and uranyl acetate, followed by two successive 15-min rinses on distilled water. Specimens were

examined and photographed in a JEOL 100CXII electron microscope (JEOL USA ... Peabody, Massachusetts).

Controls consisted of replacement of the primary antiserum with normal ... .

Isolation of neurofilament-like proteins

To demonstrate the existence of neurofilament-like components in the sea anemone *C. gigantea,* we isolated the constituent proteins. To minimize protein contamination, food was withheld from anemones for three days before extraction. Whole anemones were cleaned of debris and weighed. Specimens were chilled for 15 min at $-70°C$ before being quickly chopped into 1-cm^3 pieces and placed in SEDTA (20 mM Na-ACES buffer, pH 7.4, 0.3 M sucrose, 2 mM EDTA) for 15 min with intermittent gentle hand agitation to remove mucus. Minced tissue was transferred to TME medium [10 mM tris(hydroxymethyl)-aminomethane (Tris) HCl, 3 mM MgCl$_2$, 2 mM K$_2$-ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), pH 8.2] and homogenized, with the aid of a Teflon-glass homogenizer, in the presence of protease inhibitors [10 μg/ml leupeptin, 76.8 nM aprotinin, 0.7 μM pepstatin, 0.83 mM benzamide, 0.23 mM phenylmethanesulfonyl fluoride (PMSF), and 1 mM iodoacetamide] at 4°C. The homogenate was centrifuged at $9000 \times g$ (SS34 rotor) for 2 h at 0°C and the supernatant (S1) reserved. The pellet was successively washed four times in ice cold TME with a brief centrifugation after each wash at $9000 \times g$ for 15 min at 0°C. Because of the difficulty encountered in solubilizing cytoskeletal components in Triton X-100 in early experiments, all subsequent procedures utilized sodium dodecyl sulfate (SDS). Pelleted material was resuspended in TME containing 2% SDS, homogenized in a Teflon-glass homogenizer, and centrifuged at $9000 \times g$ for 2 h at 0°C. The supernatant (S2) was removed and stored at $-70°C$. The pellet (P2) was washed in TME and re-extracted in SDS. Any remaining insoluble material was removed by centrifugation at $9000 \times g$ for 2 h at 0°C, and the supernatant (S3) was stored at $-70°C$. Protein content was determined by the method of Bradford (1976).

Control procedures consisted of extraction of rat brain as described above.

Absorption of the neurofilament-like extract

In several experiments, polyclonal NF 200 antibodies were absorbed with the washed resuspension of neurofilament-like protein (S2, above) from which the SDS had been removed (Suzuki and Terada, 1988) prior to use. The preparation, consisting of the extract of neurofilament-like proteins and the added antibodies, was placed at 4°C for 24 h with gentle stirring. The supernatant was

decanted after centrifugation at $9500 \times g$ for 2 h at 0°C and was used for immunostaining.

SDS-polyacrylamide gel electrophoresis and Western blotting

Proteins were electrophoresed in the presence of SDS on 12.5% and 4/20% gradient gels by the method of Laemmli (1970) and transferred to nitrocellulose sheets. Western blotting was performed by the method of Towbin *et al.* (1979). In brief, blots were processed through incubations with blocking solution (5% skim milk in 0.1 M PBS/0.1% Tween-20) for 12 h at 4°C, and immunostained with one of the following: (1) polyclonal anti-NF 200 at a 1:150 dilution of a 9.87 mg/ml stock solution (Sigma Chemical Co., St. Louis, Missouri); (2) monoclonal antibody generated to mouse NF 200 (phosphorylated and nonphosphorylated forms; Clone N52) at a 1:150 dilution of the stock solution (Sigma Chemical Co., St. Louis, Missouri); (3) monoclonal anti-microtubule-associated protein-2 (anti-MAP-2) at a 1:2000 dilution of a 2 mg/ml stock solution (clone AP20; Boehringer Mannheim Corp., Indianapolis, Indiana); (4) monoclonal anti-tau microtubule-associated protein (anti-tau) at a 1:100 dilution of the 1 mg/ml stock solution (clone Tau 1; Boehringer Mannheim Corp., Indianapolis, Indiana); or (5) undiluted hybridoma supernatant to an anti-IFA antibody (Antibody to Intermediate Filament Antigen; Pruss *et al.,* 1981) for 1 h at 4°C. Incubation was followed by biotinylated anti-rabbit or anti-mouse immunoglobulins and an avidin-horseradish peroxidase complex (Vector Laboratories, Burlingame, California) according to manufacturer's directions. Conjugates were visualized with a 4-chloro-1-naphthol/peroxidase substrate system (Kirkegaard & Perry, Gaithersburg, Maryland).

Results

Bodian silver stain

The Bodian method was originally developed to stain neural components and is known to stain NF proteins specifically. Bodian silver stain was applied to tissue sections and to SDS polyacrylamide gels after electrophoresis. Neither method produced a visible reaction product (not shown).

Light microscopic immunocytochemistry

Figure 1 shows a transverse section of anemone tentacle stained with polyclonal antiserum raised to NF 200. These antibodies exhibit wide species cross-reactivity (Chemicon International, Temecula, California, Sigma Chemical Co., St. Louis, Missouri). A large proportion of the nickel-enhanced immunoreactive product was localized to an area above the mesoglea known to contain a dense plexus
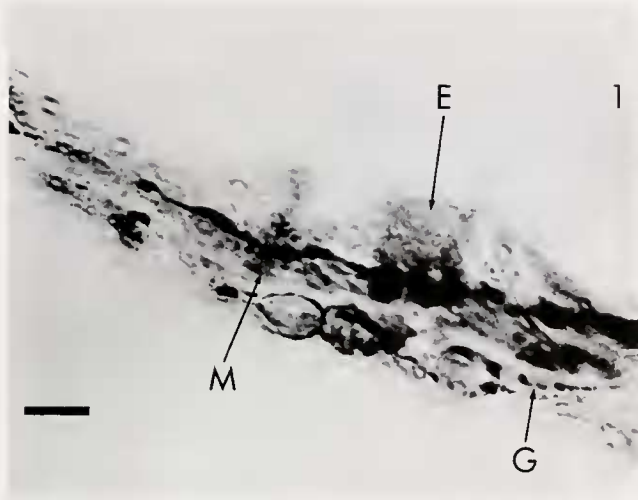
Figure 1. Light microscopy. Cross-section of a tentacle of *Condylactis gigantea* showing specific nickel-enhanced immunostain with polyclonal NF 200 antisera. The product is localized to an area above the muscle layer and mesoglea that contains the neural plexus. Reaction product is also seen in the gastrodermis. The scale bar is 10 µm. E, epidermis; M, mesoglea; G, gastrodermis.

of nerve cell bodies and processes (C. DellaCorte, C. Fulenwider, D. Hessinger, and W.O. McClure, manuscript in prep). Immunolocalization was also seen within the gastrodermis, central to symbiotic zooxanthellae. Absorption of the antibodies with an extract of *C. gigantea* neurofilament-like protein removed this reactivity. Replacement of the primary, specific antibody with normal serum, or deletion of the secondary antibody, also eliminated the appearance of product.

Electron microscopic immunocytochemistry

Immunocytochemical localization within the tissues of *C. gigantea* was examined further by electron microscopy. With the chosen protocol, we were able to preserve antigenicity. Cytoskeletal morphology could not be fully maintained, however. Immunogold labeling was predominately localized within the processes of the neural plexus (Fig. 2) above the layer of myocytes. Immunogold labeling displayed the same relative pattern in all cellular neural compartments considered. No appreciable number of gold particles were observed over non-neural tissues. The specificity of labeling was assessed with controls: sections adjacent to those immunolabeled with anti-NF 200 were reacted with normal serum. The control sections lacked the labeling described above, or showed only extremely sparse and randomly scattered distribution of gold particles, demonstrating low background. These observations provide additional evidence that the immunoreactive material is located in the neurons of *C. gigantea*.

Gel electrophoresis and Western blotting

To further demonstrate the existence of neurofilament-like proteins in this species, we probed Western blots of Triton-insoluble proteins with several specific immunological probes. Polyclonal NF 200 antiserum revealed two bands of apparent molecular weights 156,000 and 74,000 daltons (Fig. 3). A similar pattern (not shown) was found with a monoclonal antiserum known to react with an epitope present on both the phosphorylated and non-phosphorylated forms of the polypeptide in the tail domain of the NF-H subunit.

Western blots of Triton-insoluble material from *C. gigantea* were also probed with monoclonal antibodies to the microtubule-associated proteins, anti-MAP2 (Fig. 4) and anti-tau (Fig. 5), and with a monoclonal antibody (anti-IFA; Pruss *et al.*, 1981) known to bind to most of the defined major intermediate filament proteins. MAP2 isolated from rat brain consists of two major polypeptides (MAP2a and MAP2b) of similar molecular weights, approximately 275 kD, although proteolysis can result in a 35 kD domain and a major 240 kD domain (Vallee, 1980). Clear patterns of immunolabeling of the putative neurofilament bands (*i.e.*, 156,000 and 74,000 daltons) extracted from *C. gigantea* are evident in both blots probed with antibodies to MAP2 (Fig. 4) and tau (Fig. 5), as are the expected high molecular weight MAP2 protein band and the multiple 55 kD to 62 kD tau bands (Cleveland *et al.*, 1977a,b) present in the control tissues of the rat. In a similar fashion, the anti-IFA Western blot revealed two distinct bands of about 156 kD and 74 kD in the extract from the tissues of the sea anemone (Fig. 6).

Discussion

The presence of neurofilament-like proteins in the sea anemone *Condylactis gigantea* was investigated using
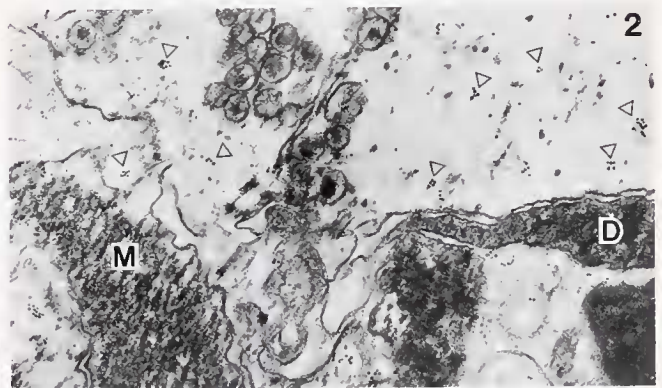

Figure 2. Transmission electron microscopy. Cross-section of a tentacle showing immuno-gold labeling of anti-NF 200 localized within the cytoplasm of the neural processes. Synaptic vesicles line a neuro-neuronal synapse. Reaction product (arrowheads) is seen within the neuronal processes. Magnification: 50,000×. M, muscle; D, desmocyte process.

215,500 —

105,100 —
69,800 —

43,300 —
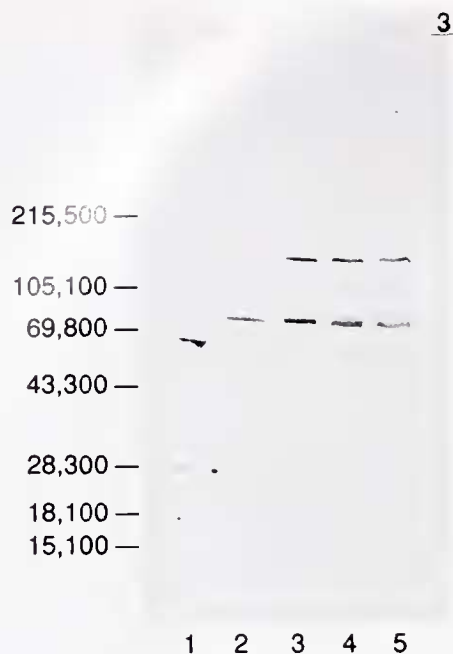
28,300 —
18,100 —
15,100 —

1   2   3   4   5

Figure 3. Western blotting. Proteins were separated on a 4/20% gradient gel and visualized with a polyclonal antibody directed against NF 200. Two bands of MW_r 156 kD and 74 kD were specifically labeled in the extract from the sea anemone *Condylactis gigantea*. Lane 1: molecular weight standards (MW_r); Lane 2: anemone S2 fraction, 5.0 μg protein per lane; Lane 3: anemone S2 fraction, 9.5 μg protein per lane; Lane 4: anemone S2 fraction, 12.0 μg protein per lane; Lane 5: anemone S2 fraction, 15.0 μg protein per lane.

several methods, including Bodian stain which binds an acidic, glutamate-rich region in the carboxy terminal of NF-L, NF-M, and NF-H (Autilio-Gambetti *et al.*, 1981). This staining technique, used to identify neurofilament proteins in many species, did not produce positive results. But other data gathered during our study suggest that neurofilament-like proteins are present in *C. gigantea*.

A number of immunological studies have shown regional phylogenetic conservation of neurofilament proteins (Chiu *et al.*, 1980; Autilio-Gambetti *et al.*, 1981; Shaw *et al.*, 1984). Immunological staining of Western blots of Triton-insoluble proteins from *C. gigantea* revealed 156,000 and 74,000 dalton molecular weight bands that correspond to those seen in published electrophoretic profiles. Explaining the presence of a low molecular weight band when using monoclonal and polyclonal antisera to NF-H is problematic, however. Many polyclonal and monoclonal antibodies reportedly recognize epitopes shared by NF-H and NF-M (Lee *et al.*, 1987; Schmidt *et al.*, 1987). Cross-reactivity between these subunits and NF-L is less well-known, although there is a report of simultaneous immunodetection of all three NF subunits (Cochard and Paulin, 1984). The presence of these immunoreactive proteins suggests that polypeptide regions

homologous to those found in other organisms are present in this species.

Immunological studies also reveal species-specific differences in the number and the molecular weight of neurofilament proteins. In birds and crocodilia, NF proteins appear to be directly homologous to the neurofilament triplet. Other reptiles express only a single high molecular weight subunit immunologically related to both NF-H and NF-M (Shaw *et al.*, 1984; Shaw, 1991). Studies in fish demonstrate between one and four (or more) major subunits (Phillips *et al.*, 1983; Lasek *et al.*, 1985; Dahl *et al.*, 1986). Neurofilaments in invertebrates, however, are more variable than their vertebrate counterparts. Two high molecular weight subunits, each about 160 kD, are found in the marine fan worm *Myxicola infundibulum* (Eagles *et al.*, 1981). Squid contain neurofilament subunits of 60, 200, and 500 kD (Roslansky *et al.*, 1980), although NF proteins with approximate weights of 60, 70, and 220 kD have recently been identified (Szaro *et al.*, 1991; Way *et al.*, 1992). *Aplysia* neurofilaments have apparent molecular weights of 60 kD and 65 kD. This nudibranch is the
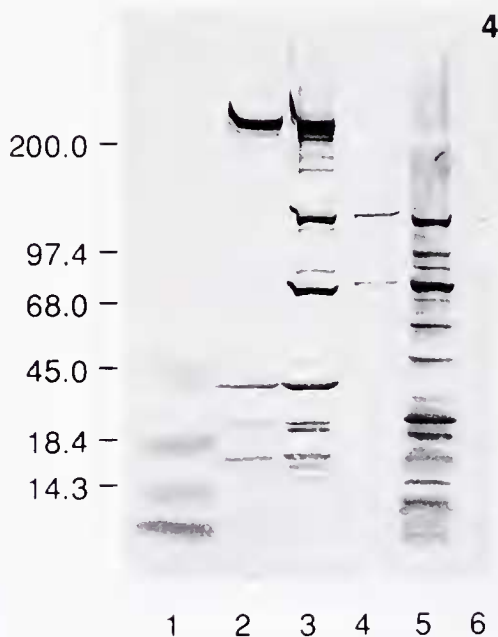

200.0 —

97.4 —

68.0 —

45.0 —

18.4 —
14.3 —

1   2   3   4   5   6

Figure 4. Western blotting. Proteins were separated on a 4/20% gradient gel and were immunoblotted with a monoclonal antibody generated to MAP2. In the protein fraction from *Condylactis gigantea*, two bands of approximate molecular weights 156 kD and 74 kD are found. Immunolocalization to protein fractions from control tissue show a large molecular weight subunit. A smaller, low molecular weight subunit, which may be due to proteolysis, is also present. Lane 1: molecular weight standards (MW_r); Lane 2: rat brain cytosolic fraction (S1), 15 μg protein per lane; Lane 3: rat brain membrane fraction (S2), 15 μg protein per lane; Lane 4: anemone S1 fraction, 10 μg protein per lane; Lane 5: anemone S2 fraction, 9.4 μg protein per lane; Lane 6: anemone S3 fraction, 10 μg protein per lane.
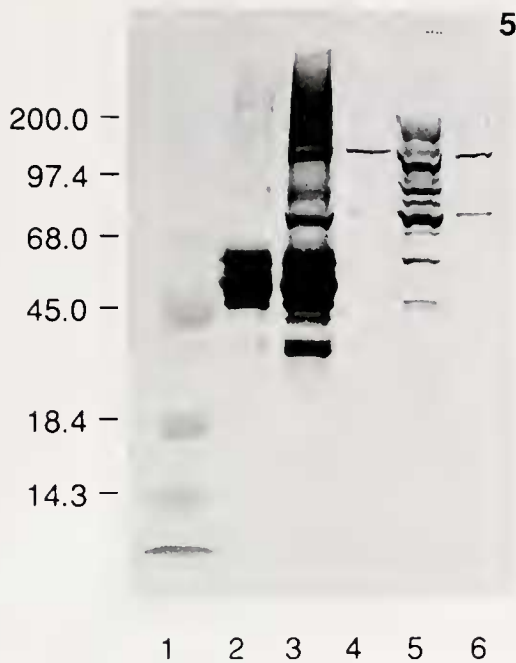
Figure 5. Western blotting. Proteins were separated on a 12.5% gel and bands visualized using monoclonal anti-tau antibody. Cross-reactivity with proteins of MW_r 156 kD and 74 kD extracted from *Condylactis gigantea* is seen. Immunoblotting of protein fractions from control tissue reveals a series of bands of MW_r 55 kD to 62 kD. Lane 1: molecular weight standards (MW_r); Lane 2: rat brain cytosolic fraction (S1), 15 μg protein per lane; Lane 3: rat brain membrane fraction (S2), 15 μg protein per lane; Lane 4: anemone S1 fraction, 10 μg protein per lane; Lane 5: anemone S2 fraction, 9.4 μg protein per lane; Lane 6: anemone S3 fraction, 10 μg protein per lane.

only species investigated to date that does not express a subunit with a molecular weight greater than 100 kD and whose NFs lack an affinity for Bodian stain (Lasek *et al.*, 1985). The presence of only two neurofilament-like proteins in *C. gigantea* may be a reflection of species-specificity.

More evidence for the presence of neurofilament-like epitopes was provided by absorption of the extract containing the neurofilament-like proteins with neurofilament antibodies and by immunoblotting with an antibody, anti-IFA, known to recognize a common antigenic determinant. In many species, anti-IFA has identified the presence of intermediate filaments, although immunoreactivity was not detected in the single species of Cnidaria investigated by others (Pruss *et al.*, 1981; Bartnik *et al.*, 1985; Shaw, 1991). Our immunoblot results with anti-IFA, however, suggest that *C. gigantea* does, in fact, contain the evolutionarily highly conserved epitope. Recognition of this sequence within the rod portion of the filament has, in many cases, been used to identify intermediate filaments (Shaw, 1991).

Microtubules are primary cytoskeletal elements that form polymers with neurofilaments in the nerve cells of

many species (Wuerker and Palay, 1969; Hirokawa, 1982). These polymers contain a variety of microtubule-associated proteins (MAPs), including MAP2 and the closely related protein family, tau (Borisy *et al.*, 1975; Weingarten *et al.*, 1975; Cleveland *et al.*, 1977a,b). Antibodies generated to MAP2 and tau cross-react with neurofilament proteins (Kosik *et al.*, 1988; Shaw, 1991). Investigations suggest similarities in sequence and the presence of heavily phosphorylated regions as one explanation for the cross-reactive properties. Tau from rat brain contains four internal repeats consisting of a "Lys-Ser-Pro-Val" motif in the COOH-terminal domain similar to the numerous Lys-Ser-Pro (KSP) repeats found in neurofilament (Kanai *et al.*, 1989; Shaw, 1991. Sequences of MAP2 also demonstrate carboxy-terminal repeats that are highly homologous to those found in tau (Lewis *et al.*, 1988). In addition, some tau isoforms contain amino-terminus regions rich in proline and charged amino acid residues (Goedert *et al.*, 1991. The well-characterized Tau 1 antibody used in this study recognizes an amino terminus epitope located between Pro 189 and Gly 207. This sequence is suggested to be similar to the carboxy-terminus of NF-M (Kosik *et al.*, 1988; Goedert *et al.*, 1991). The antibody is not known to react, however, when Ser 199 and Ser 202 are phosphorylated (Riederer and Binder, 1994). The MAP2 an-
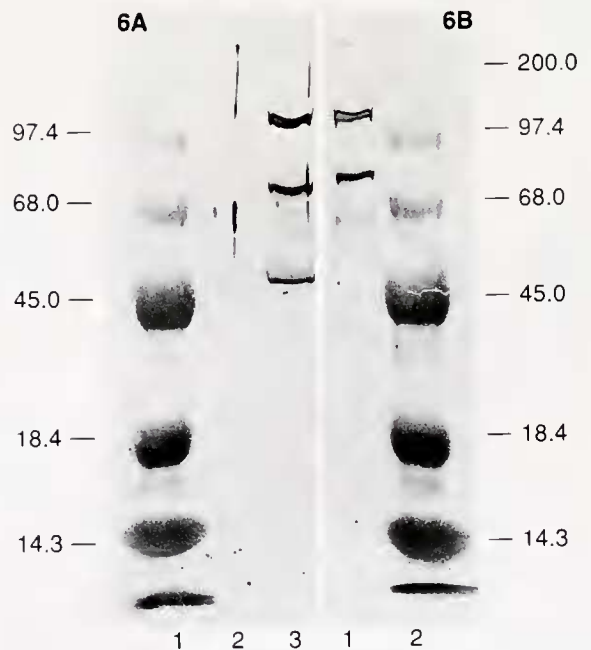

Figure 6. Western blotting. Proteins were electrophoresed on a 12.5% gel and reacted with anti-IFA, resulting in the visualization of two bands of MW_r 156 kD and 74 kD in the extract from *Condylactis gigantea*. (A) Lane 1: molecular weight standards (MW_r); Lane 2: rat brain cytosolic fraction (S1), 15 μg protein per lane; Lane 3: rat brain membrane fraction (S2), 15 μg protein per lane; (B) Lane 1: anemone S2 fraction, 9.4 μg protein per lane; Lane 2: molecular weight standards (MW_r).

tibody, clone AP20, has only recently been mapped to a
carboxy terminus encompassing amino acids 997-1332
(L. Binder, pers. comm.), a region suggested to contain
sequences closely related to those found in NF-H, NF-M,
and tau proteins (Shaw, 1989). The presence of these con-
served regions suggests evolutionary relationships and may
account for the immunological similarity seen in a num-
ber of studies, including ours.

Considering the well-documented description of a dif-
fuse nerve net in cnidarians, we were surprised to find
little immunostaining throughout the epidermis and gas-
trodermis of the tentacle using anti-NF200. Most of the
reaction product was localized within an epidermal neural
plexus and, to a lesser extent, to components within the
gastrodermis. This pattern was reversed when tissues were
immunostained with a polyclonal antiserum generated to
NF 150 (C. DellaCorte, unpub. ob.). Selective reactivity
of the antisera (*i.e.*, immunodetection of specific subsets
of neural components) was not expected but may be the
result of several factors including cell size, cell type, pres-
ence or lack of post-translational modifications, or the
cross-reactivity of the antisera. Many regions in mam-
malian nervous systems show strong NF staining for some
neuronal cell types and partial or complete absence of
immunoreactivity in others. This is particularly true for
small neurons (Sharp *et al.*, 1982; Trojanowski *et al.*,
1986). In addition to other factors, the type and size of
cells in the nervous system of *Condylactis gigantea* may
affect the presence and the extent of immunoreactivity in
this invertebrate.

The present data suggest that neurons in this anthozoan
may contain proteins with molecular properties similar
to those of higher species. Although various assumptions
about these neurofilament-like proteins may be made, no
definitive conclusions can yet be drawn. Knowledge of
the primary structure of these early metazoan proteins
will provide insight into the functional constraints of pro-
tein sequences during evolution.

Literature Cited

Autilio-Gambetti, L., M. E. Velasco, J. Sipple, and P. Gambetti.
1981. Immunochemical characterization of antisera to rat neuro-
filament subunits. *J. Neurochem.* 37: 1260-1265.

Bartnik, E., M. Osborn, and K. Weber. 1985. Intermediate filaments
in non-neuronal cells of invertebrates: isolation and biochemical
characterization of intermediate filaments form the esophageal epi-
thelium of the mollusk *Helix pomatia. J. Cell Biol.* 101: 427-440.

Berod, A., B. K. Hartman, and J. F. Pujol. 1981. Importance of fixation
in immunohistochemistry: Use of formaldehyde solutions at variable
pH for the localization of tyrosine hydroxylase. *J. Histochem. Cy-
tochem.* 29: 844-885.

Bodian, D. 1936. A new method for staining nerve fibers and nerve
endings in mounted paraffin sections. *Anat. Rec.* 65: 89-97.

Borisy, G. G., J. M. Marcum, J. B. Olmstead, D. B. Murphy, and
K. A. Johnson. 1975. Purification of tubulin and associated high
molecular weight proteins from porcine brain and characterization
of microtubule assembly in vitro. *Ann. NY Acad. Sci.* 253: 107-132.

Bradford, M. M. 1976. A rapid and sensitive method for the quanti-
tation of microgram quantities of protein utilizing the principle of
protein-dye binding. *Anal. Biochem.* 72: 248-254.

Brown, S., W. Levinson, and J. A. Spudich. 1976. Cytoskeletal elements
of chick embryo fibroblasts revealed by detergent extraction. *J. Su-
pramol. Struct.* 5: 119-130.

Bullock, T. H., and G. A. Horridge, eds. 1965. Coelenterata and Cten-
ophora. Pp. 459-534 in *Structure and Function in the Nervous Sys-
tems of Invertebrates*, Vol 1. W. H. Freeman Press, San Francisco.

Chiu, F. C., B. Korey, and W. T. Norton. 1980. Intermediate filaments
from bovine, rat and human CNS: Mapping analyses of the major
proteins. *J. Neurochem.* 34: 1149-1159.

Clark, G. ed. 1981. Bodian's protargol method. Pp. 152-154 in *Staining
Procedures.* Williams and Wilkins, Baltimore, MD.

Cleveland, D. W., S. Hwo, and M. W. Kirschner. 1977a. Purification
of tau, a microtubule-associated protein that induces assembly of
microtubules from purified tubulin. *J. Mol. Biol.* 116: 207-225.

Cleveland, D. W., S. Hwo, and M. W. Kirschner. 1977b. Physical and
chemical properties of purified tau factor and the role of tau in mi-
crotubule assembly. *J. Mol. Biol.* 116: 227-247.

Cochard, P., and D. Paulin. 1984. Initial expression of neurofilament
and vimentin in the central and peripheral nervous system of the
mouse embryo in vivo. *J. Neurosci.* 4: 2080-2094.

Cooke, P. H., and R. H. Chase. 1971. Potassium chloride insoluble
myofilaments in vertebrate smooth muscle cells. *Exp. Cell Res.* 66:
417-425.

Dahl, D., C. J. Crosby, and A. Bignami. 1986. Neurofilament proteins
in fish: a study with monoclonal antibodies reacting with mammalian
NF 150K and NF 200K. *J. Comp. Neurol.* 250: 399-402.

Daniels, M. P., and F. C. Huneeus. 1973. Fine structural changes in
neurons and nerve fibers associated with colchicine inhibition of nerve
fiber formation in vitro. *J. Cell Biol.* 58: 463-470.

Eagles, P. A. M., D. S. Gilbert, and A. Maggs. 1981. The location of
phosphorylation sites and Ca^{2+}-dependent proteolytic cleavage sites
on the major neurofilament polypeptides from *Myxicola infundib-
ulum. Biochem. J.* 199: 101-111.

Gambetti, P., L. Autilio-Gambetti, and S. Ch. Papasozomenos.
1981. Bodian's silver method stains neurofilament polypeptides.
Science 213: 1521-1522.

Geisler, N., and K. Weber. 1982. The amino acid sequence of chicken
muscle desmin provides a common structural model for intermediate
filament proteins. *EMBO J.* 1: 1649-1656.

Goedert, M., R. A. Crowther, and C. C. Garner. 1991. Molecular char-
acterization of microtubule-associated proteins tau and MAP2. *Trends
Neurosci.* 5: 193-199.

Hanukoglu, I., and E. V. Fuchs. 1983. The cDNA sequence of a type II cytoskeletal keratin reveals constant and variable structure domains among keratins. *Cell* 33: 915–924.

Hirokawa, N. 1982. The crosslinker system between neurofilaments, microtubules and membranous organelles in frog axons revealed by quick freeze, freeze-fracture, deep-etching method. *J. Cell Biol.* 94: 129–142.

Kanai, Y., R. Takemura, T. Ohshim, H. Mori, Y. Ihara, M. Yanagisawa, T. Masaki, and N. Hirokawa. 1989. Expression of multiple tau isoforms and microtubule bundle formation in fibroblasts transfected with a single tau cDNA. *J. Cell Biol.* 109: 1173–1184.

Kaufmann, E., N. Geisler, and K. Weber. 1984. SDS-PAGE strongly overestimates the molecular masses of the neurofilament proteins. *FEBS Lett.* 170: 81–84.

Kosik, K. S., L. D. Orecchio, L. Binder, J. Q. Trojanowski, V. M.-Y. Lee, and G. Lee. 1988. Epitopes that span the tau molecule are shared with paired helical filaments. *Neuron* 1: 817–825.

Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 227: 680–685.

Lasek, R. J., N. Krishnan, and I. R. Kaiserman-Abramof. 1979. Identification of the subunit proteins of 10 nm neurofilaments isolated from axoplasm of Squid and *Myxicola* giant axons. *J. Cell Biol.* 82: 336–346.

Lasek, R. J., L. Phillips, M. J. Katz, and L. Autilio-Gambetti. 1985. Function and evolution of neurofilament proteins. *Ann. N.Y. Acad. Sci.* 455: 462–478.

Lee, V. M.-Y., M. J. Carden, W. W. Schlaepfer, and J. Q. Trojanoski. 1987. Monoclonal antibodies distinguish several differentially phosphorylated states of the two largest neurofilament subunits (NF-H and NF-M) and demonstrate their existence in the normal nervous system of adult rats. *J. Neurosci.* 7: 3474–3488.

Lewis, S. A., D. Wang, and N. J. Cowan. 1988. Microtubule-associated protein MAP2 shares a tubulin-binding motif with tau protein. *Science* 242: 936–939.

Moon, H. M., T. Wisniewski, P. Merz, J. De Martini, and H. M. Wisniewski. 1981. Partial purification of neurofilament subunits from bovine brains and studies on neurofilament assembly. *J. Cell Biol.* 89: 560–567.

Phillips, L. L., L. Autilio-Gambetti, and R. J. Lasek. 1983. Bodian's silver method reveals molecular variation on the evolution of neurofilament proteins. *Brain Res.* 278: 219–223.

Pruss, R. M., R. Mirsky, and M. C. Raff. 1981. All classes of intermediate filaments share a common antigenic determinant defined by a monoclonal antibody. *Cell* 27: 419–428.

Riederer, B. M., and L. I. Binder. 1994. Differential distribution of tau proteins in developing cat cerebellum. *Brain Res. Bull.* 33: 155–161.

Roslansky, P. F., A. Cornell-Bell, R. V. Rice, and W. J. Adelman. 1980. Polypeptide composition of squid neurofilaments. *Proc. Natl. Acad. Sci. USA* 77: 404–408.

Schmidt, M. I., M. J. Carden, V. M.-Y. Lee, and J. Q. Trojanoski. 1987. Phosphate dependent and independent neurofilament epi-topes on the axonal swellings of patients with motor neuron disease and controls. *Lab Invest* 56: 282–294.

Scott, D., K. E. Smith, B. J. O'Brien, and K. J. Angelides. 1985. Characterization of mammalian neurofilament triplet proteins, subunit stoichiometry and morphology of native and reconstituted filaments. *J. Biol. Chem.* 260: 10736–10747.

Sharp, G. A., G. Shaw, and K. Weber. 1982. Immunoelectronmicroscopical localization of the three neurofilament proteins along neurofilaments in cultured dorsal root ganglion cells. *Exp. Cell Res.* 137: 403–413.

Shaw, G. 1989. Identification of previously unrecognized sequence motifs at the extreme carboxyterminus of the neurofilament subunit NF-M. *Biochem. Biophys. Res. Comm.* 162: 294–299.

Shaw, G. 1991. Neurofilament proteins. Pp. 185–214 in *The Neuronal Cytoskeleton*, R. D. Burgoyne, ed. Wiley-Liss, Inc., New York.

Shaw, G., E. Debus, and K. Weber. 1984. The immunological relatedness of neurofilament proteins of higher vertebrates. *Eur. J. Cell Biol.* 34: 130–138.

Steinert, P. M., R. H. Rice, D. R. Roop, B. L. Trus, and A. C. Steven. 1983. Complete amino acid sequence of a mouse epidermal keratin subunit and implications for the structure of intermediate filaments. *Nature* 302: 794–800.

Steinert, P. M., and D. R. Roop. 1988. Molecular and cellular biology of intermediate filaments. *Annu. Rev. Biochem.* 57: 593–625.

Suzuki, H., and T. Terada. 1988. Removal of dodecyl sulfate from protein solution. *Anal. Biochem.* 172: 259–263.

Szaro, B. G., H. C. Pant, J. Way, and J. Battey. 1991. Squid low molecular weight neurofilament proteins are a novel class of neurofilament protein. *J. Biol. Chem.* 266: 15035–15041.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76: 4350–4354.

Trojanowski, J. Q., N. Walkenstein, and V. M.-Y. Lee. 1986. Expression of neurofilament subunits in neurons of the central and peripheral nervous system; an immunohistochemical study with monoclonal antibodies. *J. Neurosci.* 6: 650–660.

Vallee, R. B. 1980. Structure and phosphorylation of microtubule-associated protein 2 (MAP2). *Proc. Natl. Acad. Sci. USA* 77: 3206–3210.

Wang, E., D. Fischman, R. K. H. Liem, and T-T. Sun. eds. 1985. *Intermediate Filaments. Ann. N.Y. Acad. Sci.* 455: 1–832.

Way, J., M. R. Hellmich, H. Jaffe, G. Szaro, H. C. Pant, H. Gainer, and J. Battey. 1992. A high-molecular-weight squid neurofilament protein contains a lamin-like rod domain and a tail domain with Lys-Ser-Pro repeats. *Proc. Natl. Acad. Sci. USA* 89: 6963–6967.

Weingarten, M. D., A. H. Lockwood, S. Hwo, and M. W. Kirschner. 1975. A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. USA* 72: 1858–1862.

Wuerker, E. B., and S. L. Palay. 1969. Neurofilaments and microtubules in anterior horn cells of the rat. *Tissue Cell* 1: 387–402.